REMARKS

Status of the claims

Claims 1-18 are pending in the present application. Claims 1-7 and 10-14 are withdrawn per the Restriction Requirement issued on September 21, 2004. Claims 8, 9 and 15 are amended herein. Claims 16-18 are newly added. Support for the amendments to claims 8 and 15 and for new claims 16-18 may be found on pages 20 and 21 of the specification. No new matter has been added with these amendments or new claims.

Objections to the specification

The Examiner objects to several formal issues with the specification. The specification has been amended, as indicated above, to address these issues. No new matter has been introduced with the amendments. In addition, as one objection, the Examiner indicates that there are sequences disclosed in Figures 1-12 that do not contain sequence identifiers. The Examiner's attention is directed to the Preliminary Amendment submitted to the USPTO on March 1, 2002, in which the figure descriptions beginning at page 12, line 15 of the specification were amended to insert sequence identifiers.

Priority Claim under 35 U.S.C.§120

The Examiner advises in Item 4, page 4 of the Office Action that the Applicant has not complied with the requirements for a claim to priority under 35 U.S.C. §120 because the specification has not been amended to reference the priority application(s). Applicants note that specification was appropriately amended through an amendment on the filing transmittal when the application was filed on February 28, 2002.

Objections to the claims

Claim 9 has been objected to for reciting "an" instead of "the." Claim 9 has been amended to address this issue.

Rejections under 35 U.S.C.§112, 1st paragraph

Claims 8, 9 and 15 have been rejected under 35 U.S.C. §112, 1st paragraph for lacking enablement and written description. The Examiner asserts that the specification enables/describes treating GVHD with antagonists that are Fas-derived antagonists and anti-Fas antibodies or anti-Fas ligand antibodies. The Examiner asserts that the specification does not provide adequate enablement for the following:

i) antisense oligonucleotides for mRNA or Fas/Fas ligand genes

ii) a substance that interacts with the intracellular domain of Fas; or

iii) an ICE inhibitor.

The Examiner further asserts that the specification fails to provide adequate written description of ii) a substance that interacts with the intracellular domain of Fas; and iii) an ICE inhibitor.

The claims have been amended to define the antagonist as Fas antagonist "providing activity to interact with the extracellular domain of the Fas ligand or the extracellular domain of Fas, an antisense oligonucleotide of Fas or an antisense oligonucleotide of Fas ligand." Thus, rejection for lack of written description is moot. In addition, the rejection for lack of enablement with regard to a substance that interacts with the intracellular domain of Fas and with regard to an ICE inhibitor is moot.

Applicants traverse the rejection with regard to an asserted lack of enablement for antisense oligonucleotides and withdrawal thereof is respectfully. The Examiner has rejected the claims for lacking enablement, but not for lacking written description, for antisense oligonucleotides. In support of rejection for lack of enablement, the Examiner relies on several articles dating from 1995, roughly one year prior to the earliest priority date of the

application, for showing the difficulties with gene therapy at that time.

However, at the time of the invention, i.e. October of 1996, the use of antisense oligonucleotides as an *in vivo* therapeutic agent was well-know to those of ordinary skill in the art. Attached hereto are three references (Refs. 1-3) which exemplify the use of antisense oligonucleotides as a therapeutic agent. The references evidence that administration methods for gene therapy were already well-known at the time of the invention. As such, it was not necessary for the enablement of the invention for the Applicants to include a detailed description in the specification of how to use anti-sense oligonucleotides.

Applicants further note that the prophylactic or therapeutic method of treating graft versus host disease (GVHD) of the present invention using Fas or Fas ligand antisense oligonucleotides as an active component is not directed to a general method of gene therapy, but rather for a method of using Fas or Fas ligand antisense oligonucleotides as an active component in a patient who needs the therapy. An example of a sequence of Fas antisense nucleotides that may be used as the active component was described in WO95/13293 before the filing date of the present application. See page 30, lines 18-24 of the specification.

Reference 1, Nature Medicine (1997, August) Vol. 3, page 900, discloses an experimental model with mouse heart transplant, wherein antisense phosphorothioate oligonucleotides were administered and in which the expression of cdk2 mRNA was inhibited along with neointimal formation and VCAM-1 expression. See Table 1 of Reference 1. Reference 1 further teaches methods of administration using incorporation into liposomes.

Reference 2, PNAS (1996, October) Vol. 93, page 11421, discloses the use of viral liposomes in gene therapy. Specifically, Reference 2 describes that the use of HVJ-liposomes enabled the *in vivo* transfer to each organ. See Table 1 of Reference 2.

Reference 3, BMJ (1997, January) Vol. 314, page 126, pertains to the pharmaceutical application of molecular genetics. Specifically, in the section entitled "Inhibiting gene expression may also be therapeutic" of Reference 3, the pharmaceutical application of antisense olignucleotides is described. For example, Reference 3 describes that fibrocellular intimal hyperplasia underlying restenosis of antheromatous vessels is blocked by c-myb antisense.

Thus, contrary to the assertion of the Examiner, the present invention is fully enabled for the use of antisense oligonucleotides as recited in the claims. Withdrawal of the rejection is, therefore, respectfully requested.

Rejections for under 35 U.S.C. §102

The claims have been rejected under 35 U.S.C. §102 as being anticipated by Palmer et al. U.S. 5,776,718; Du et al. BBRC 226:595-600 (September 24, 1996); or Braun et al. J. Exp. Med. 183:657-661 (February 1996).

Palmer et al. is relied on for teaching ICE inhibitors for treating GVHD. The claims no longer encompass the use of ICE inhibitors. As such, withdrawal of the rejection is respectfully requested.

Du et al. is relied on for teaching that a hammerhead ribozyme targets both Fas-ligand and perforin mRNA and can be used for used for treating GVHD. Thus, the hammerhead ribozyme is asserted to be a Fas antagonist of the claims. The claims no longer encompass the use of intracellular antagonists that interact with Fas ligand. As such, withdrawal of the rejection is respectfully requested.

Braun et al. is relied on for disclosing experiments using a mouse GVHD model in which donor cells from Fas-L deficient mice delayed the onset of GVHD. Braun et al. is further asserted to teach the development of therapeutic strategies aimed at controlling Fas-mediated apoptosis for decreasing the risk of GVHD.

The present invention is specifically directed to a method of treating graft versus host disease (GVHD) which comprises administering an effective amount of a Fas antagonist which interacts with the extracellular domain of the Fas ligand or the extracellular domain of Fas, or with an antisense oligonucleotide of Fas or an antisense oligonucleotide of Fas ligand. While Braun et al. may suggest a role of Fas in GVHD, the reference fails to disclose the specific claimed features of the invention. As such, the method of the invention is not anticipated by Braun et al.

Rejections under 35 U.S.C.§103 - obviousness

The claims have been rejected as being obvious over Palmer et al., Du et al. and Braun et al. combined with Lynch et al. Lynch et al. is relied on for teaching anti-Fas antibodies that inhibit Fas ligand mediated apoptosis. The Examiner asserts that it would have been obvious to use the antibodies to treat GVHD because Palmer et al., Du et al. and Braun et al. teach the involvement of Fas-mediated apoptosis in GVHD.

The rejections over either of Palmer et al. or Du et al. combined with Lynch et al. are overcome with the amendments to the claims to the use of extracellular antagonists or antisense oligonucleotides.

Applicants traverse the rejection over Braun et al. combined with Lynch et al. for obviousness. As described in the specification on page 7, line 10 through page 9, line 12, the involvement of Fas-mediated apoptosis in GVHD and the possibility that a substance that specifically suppresses Fas-mediated apoptosis would be useful as a drug for treating GVHD had not been determined at the time when the application was filed.

In addition, there were multiple reports that contradict the reported findings relied upon by the Examiner, including the findings reported by Braun et al. Thus, it was not known at the time of the invention whether treating GVHD by targeting the Fas/FasL pathway should be by increasing Fas/FasL-mediated apoptosis or by inhibiting Fas/FasL-mediated apoptosis.

For example, attached hereto are relevant contemporaneous references (References 4 and 5) in the field of the invention. WO 95/32627 (WO '627, Ref. 4) discloses the use of Fas ligand to suppress lymphocyte-mediated immune responses. In particular, WO '627 discloses pharmaceutical compositions comprising Fas ligand

for treating transplant rejection. Transplant rejection is synonymous with graft versus host disease as recited in the present claims. Example 7 of WO '627 pertains to a test of whether the absence of a functional Fas ligand molecule prohibits testicular Sertoli cells from providing their immunosuppressive function.

The inventors of WO '627 report that the findings of Example 7 establish a role of Fas ligand in immunosuppression and further show that the presence of a functional Fas ligand gene protects transplanted testicular tissue from graft tissue rejection.

The role of Fas ligand in transplant rejection was further confirmed by the inventors of WO '627 in Example 8, which demonstrated that Fas ligand was an effective immunosuppressive factor, which is responsible for the immunosuppressive effect of testicular Sertoli cells. See page 29, lines 8-12 and page 30, lines 19-22. WO '672 further shows in Table 1 that transplantation to a mouse under conditions where Fas ligand is non-functional, results in a high rejection rate.

Thus, from the evidence and findings in WO '627 one skilled in the art would conclude that Fas/FasL-mediated apoptosis should be increased to treat GVHD, which is quite the opposite to the invention.

Takeda et al., Japanese Journal of Transplantation, extra edition featuring the general meeting of the Society for

Transplantation, vol. 31, page 180 (1996) (Ref. 5) teaches that Fas ligand has a protective effect against transplant rejection. The experiments of Takeda et al. show that donor mice, which have or do not have Fas ligand, respectively, when used with recipient mice which have or do not have Fas, respectively, have a high survival rate in the combination of donor mice having Fas ligand and recipient mice having Fas. From these findings it is presumed that the Fas/Fas ligand interaction acts to suppress the transplant rejection, i.e. the complete contrary finding to the invention and the suggested conclusion of Braun et al.

WO '627 and Takeda et al. were both available prior to the present application being filed. As such, one skilled in the art would have predicted the exact opposite of the invention. Based on the findings WO '627 and Takeda et al. one skilled in the art would have predicted that inhibiting the Fas ligand would not be useful in the treatment or prophylaxis of GVHD.

As discussed above, Braun et al. fails to disclose the specifically claimed method of the invention. Given the unpredictability in the field at the time of the invention, the present invention is further not obvious over Braun et al. Lynch et al. fails to make of the deficiencies of Braun et al. Lynch et al. merely teaches anti-Fas antibodies that inhibit Fas ligand mediated apoptosis. Thus, Lynch et al. fails to resolve the

unpredictability and controversy in the field at the time of the invention. As such, the present invention is not obvious over Braun et al. when considered alone or in combination with Lynch et al. Withdrawal of the rejection and allowance of the claims are therefore respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, PhD (Reg. No. 40,069) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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MAA/csm
1110-0307P
Attachments: References 1-5

Reference 1

ARTICLES

Prevention of graft coronary arteriosclerosis by antisense cdk2 kinase oligonucleotide

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Graft coronary arteriosclerosis, which limits the long-term survival of allograft recipients, is characterized by diffuse intimal thickening composed of proliferative smooth muscle cells[1,2]. We observed that messenger RNA of the cell cycle regulatory enzyme cyclin-dependent kinase (cdk) 2 kinase, which mediates smooth muscle cell proliferation, was elevated in the thickened intima of coronary arteries of murine heterotopic cardiac allografts. We studied the effects of antisense phosphorothioate oligodeoxynucleotide (ODN) against this enzyme using gene transfer mediated by a hemagglutinating virus of Japan (HVJ)–liposome complex intraluminally delivered to inhibit the intimal hyperplasia. At 30 days after transplantation, antisense cdk2 kinase ODN treatment had dramatically inhibited neointimal formation in the allografts. Expression of vascular cell adhesion molecule-1 was also suppressed by antisense cdk2 kinase. However, these effects were not observed in the sense or scrambled ODN-treated allografts. Thus, an intraluminal administration of antisense ODN directed to a specific cell cycle regulatory gene can inhibit neointimal formation after cardiac transplantation.

Cardiac transplantation as a treatment for end-stage cardiac diseases often results in accelerated graft coronary disease in long-term survivors[3]. Graft coronary arteriopathy, which is recognized as chronic rejection, can be detected in the majority of cardiac recipients within a few years following cardiac transplantation[4]. This arteriopathy is pathologically characterized by diffuse intimal thickening comprised of phenotypically modulated vascular smooth muscle cells[5]. Neointimal hyperplasia after cardiac transplantation results from vascular smooth muscle cell migration and proliferation, and many growth factors mediate neointimal formation by activating cell-cycle progression[6].

Several therapeutic trials have been performed in search of methods to prevent and treat arteriopathy, without significant success. The effects of calcium antagonists in humans were reported, but the results were clinically inconclusive[5]. Systemic administration of antibodies to the adhesion molecules CD28 and CD40 (ref. 6), or the angiotensin II receptor antagonist[7] have been reported in experimental models; however, the clinical utility of these methods has remained uncertain. Focused allograft treatment without systemic therapy is desirable to prevent systemic side effects.

It is now clear that cell growth and proliferation are dependent on the coordinated actions of cell cycle regulatory genes. Recer reports that the transcription factor E2F forms a complex wit cyclin A and cyclin-dependent kinase (cdk) 2 kinase reveal tha activation and phosphorylation of these cell cycle regulator genes are critical to the process of cell growth and proliferation The enzyme cdk2 kinase plays an important role in cell trans tion through the G_1–S phase, whereas cell division cycle (cdc) kinase is important in transition through the G_2–M phase Recent progress in the regulation of cell-cycle progression has r inforced the importance of the G_1–S phase in the process of ce proliferation[8,9]. Morishita *et al.* reported that intimal hyperplas after vascular balloon injury was inhibited by antisense cdk2 k nase[11] or antisense cdc2 kinase and proliferating-cell nuclea antigen (PCNA) oligodeoxynucleotides (ODNs)[12] using th hemagglutinating virus of Japan (HVJ)–liposome method[13] However, the role of these cell cycle regulatory genes in th pathophysiology of graft coronary arteriosclerosis has not bee investigated.

We hypothesized that cdk2 kinase plays a critical role in gra coronary arteriopathy. This hypothesis was tested in a murin model of heterotopic heart transplantation using BALB/c (H–2 donors and C3H/He (H–2^b) recipients. All recipients received i traperitoneal administration of FK506 (0.1 mg/kg) daily, becau allografts are acutely rejected within 10 days without administr tion of immunosuppressants. Mice were killed at days 14, 30 an 60 after transplantation for immunohistological and molecul biological studies of the grafted hearts.

In situ reverse transcriptase polymerase chain reaction (R PCR) was used for detection of cdk2 kinase messenger RNA-pos tive cells in the graft coronary arteries[14]. The cdk2 kina mRNA-expressing cells were observed in the thickened intima the allograft arteries at day 14 after transplantation, but not i the normal coronary arteries of untransplanted hearts or is grafted hearts.

The antisense method is an innovative and attractive strate to block the transcription or translation of specific genes[15]. T HVJ–liposome method, in which liposome packaged ODN coated with inactivated HVJ, has been demonstrated to increa efficiency of cellular uptake of ODN (ref. 15). To clarify the ef cacy and stability of gene transfer to heart grafts using t HVJ–liposome method, fluorescein isothiocyanate (FITC)-l beled scrambled ODN was transfected into murine cardiac all grafts. HVJ–liposome complex-coated ODN was infused into t coronary arteries of the harvested donor hearts which were inc

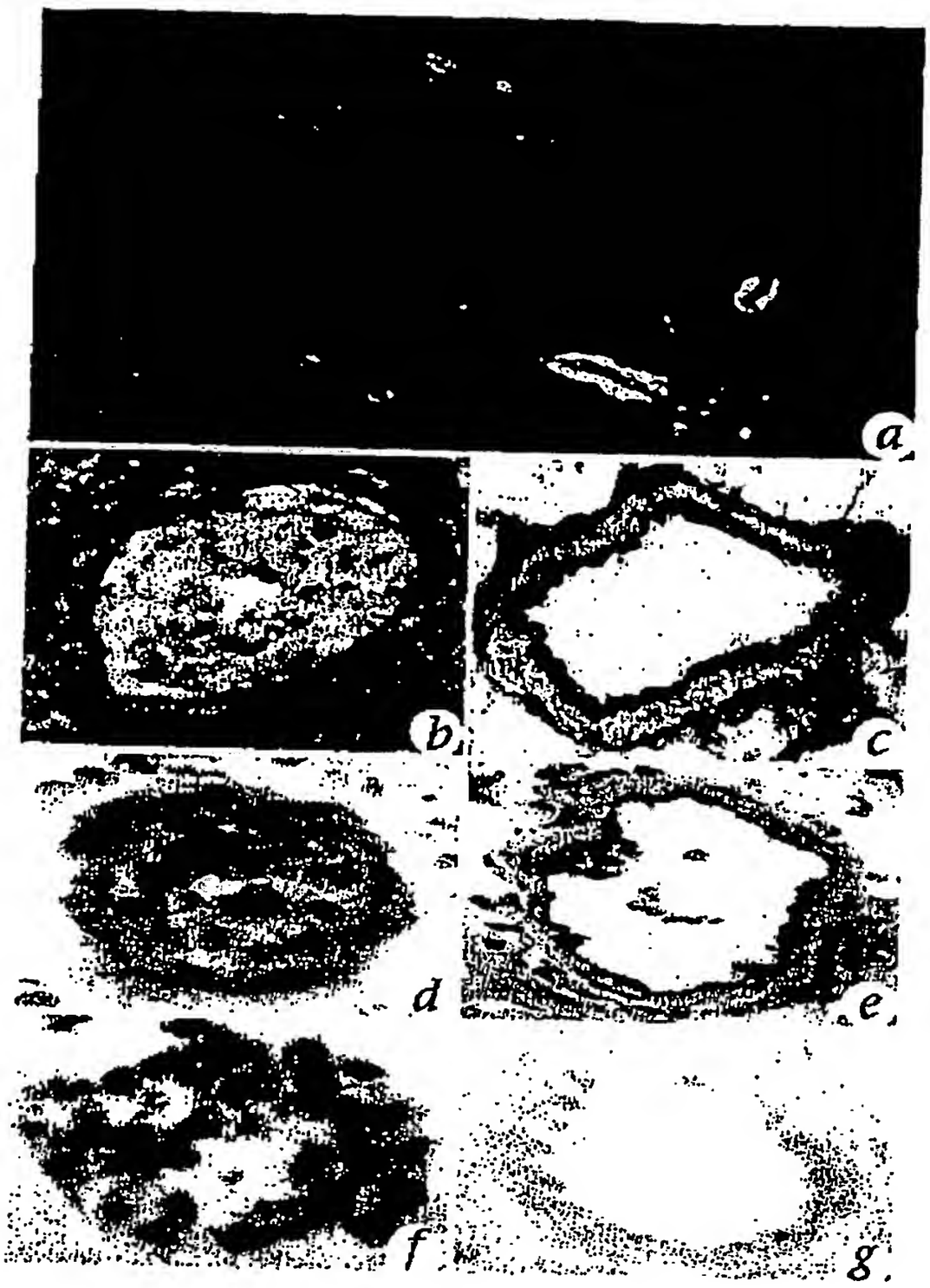

Fig. 1 *a*, The result of *in vivo* transfection of FITC-labeled ODN. Transfer o FITC-labeled phosphorothioate ODN using the HVJ method resulted ir widespread distribution of fluorescence in medial vascular and myocardia. cells at 14 days after transfection. (Original magnification, ↔40.) *b–g*, Allograft arteries at day 30 after transplantation. *b* and *c*, Coronary arteries stained with EvG to demonstrate intimal thickening. Antisense ODN transfection (*c*) limited intimal thickening, whereas untransfected arteries (*b*) show severe intimal thickening. *d* and *e*, VCAM-1 expression revealed by immunohistochemistry. *f* and *g*, Cdk2 kinase mRNA expression detected by *in situ* RT-PCR. *d* and *f*, Allograft arteries from mice treated daily with FK506 alone; *e* and *g* are those transfected with antisense cdk2 kinase ODN plus the FK506 treatment. Cdk2 kinase and VCAM-1 were strongly and diffusely expressed in the thickened intima of the allograft arteries from recipients without ODN transfection, whereas their expressions were weak in the allografts transfected with the antisense ODN. (Original magnification, ×400.)

bated for 10 min on ice, and then the donor hearts were transplanted into recipient mice. At 14 days after transfection, widespread distribution of fluorescence in cell nuclei of the medial vascular smooth muscle cells and myocardial cells was observed (Fig. 1*a*). However, these findings were not observed at day 30. This was similar to results seen in rat carotid arteries[11] and other organs[16] transfected with FITC-labeled ODN.

To test the effect of antisense cdk2 kinase ODN on the inhibition of arterial neointimal formation, we performed *in vivo* gene transfer of the ODN into murine cardiac allografts using the HVJ–liposome method. Sense cdk2 kinase ODN and scrambled ODN transfers were made for comparison. Control allografts received no ODN transfer. The sequences of ODN against human cdk2 kinase used in this study are described elsewhere[11]. Donor hearts were infused with 0.2 ml HVJ–liposome complex solution from the descending aorta; the hearts were then immediately transplanted into recipients[17]. FK506 was administered to the recipients to suppress acute cellular rejection as mentioned above, and allografts were removed at day 30 or 60. We have already shown that this amount of daily FK506 is effective in suppressing acute rejection of transplanted hearts in this combination of mice for a prolonged period of time; however, heavy intimal thickening of coronary arteries develops within 60 days[18]. Serial sections were stained with Elastica van Gieson (EvG). The lumen, occluded by intimal thickening of the arteries, was analyzed using a computer-assisted digitizer, and the percentage of intimal thickening was calculated as described before[19].

As shown in Table 1, intima of allograft coronary arteries were thickened in the mice treated with FK506 alone at day 30 after transplantation (Fig. 1*b*). The arteries with sense cdk2 kinase ODN or scrambled ODN treatment had thickened intima indistinguishable from those in the group treated with FK506 alone. However, treatment with antisense ODN to cdk2 kinase dramatically reduced arterial intimal thickening (Fig. 1*c*) ($P < 0.05$ vs. other groups) at day 30. All allografts kept beating for more than 30 days; limited myocardial cell infiltration was observed, and rejection scores did not differ among the groups.

The process of inflammatory cell emigration into tissues involves the expression of adhesion molecules on the endothelium. Transendothelial migration and positioning further contribute to smooth muscle cell proliferation. The expression of vascular cell adhesion molecule (VCAM)-1 on the vascular endothelium increases in conjunction with atherogenesis[20]. Therefore, the expression of VCAM-1 provides a molecular marker for early arteriosclerosis in chronic cardiac rejection. VCAM-1 was strongly and diffusely expressed in the thickened intima of the allografts from the recipients with FK506 alone, scrambled ODN, or sense cdk2 kinase ODN, whereas the expression was weak in the allografts from mice that received antisense

Table 1 Pathological findings of allografts at day 30

Treatment of allografts	No. of grafts	No. of arteries	Luminal occlusion(%)	VCAM-1 expression (0–3)	cdk2 mRNA expression (0–3)	Myocardial rejection score (0–3)
Antisense ODN	9	36	14.3 ± 5.3*	1.0 ± 0.6*	0.5 ± 0.8*	0.4 ± 0.5
Sense ODN	7	28	70.5 ± 20.8	2.3 ± 0.7	2.2 ± 0.8	0.4 ± 0.5
Scrambled ODN	6	26	60.6 ± 20.6	2.7 ± 0.5	2.2 ± 0.7	0.3 ± 0.5
No ODN	6	26	62.3 ± 20.9	2.4 ± 0.8	2.0 ± 0.7	0.3 ± 0.5

All allografts continued beating for 30 days. Scoring of the intensity of VCAM-1 and cdk2 kinase mRNA expression was as follows: 0 = no visible staining, 1 = few cells with faint staining, 2 = moderate staining, and 3 = intense diffuse staining. Scoring of the myocardial rejection was as follows: 0 = no cell infiltration, 1 = faint and limited cell infiltration, 2 = moderate cell infiltration, and 3 = severe and diffuse cell infiltration with myocyte damage. The mean score derived from two independent reviewers was employed.
*$P < 0.05$ vs. other groups.

ODN to cdk2 kinase (Fig. 1, *d* and *e*). The expression of intercellular adhesion molecule-1 was similarly affected (data not shown). The cdk2 kinase mRNA was not detected in the arteries of the allografts treated with antisense cdk2 kinase ODN, but was expressed throughout the allografts from the other treatment groups (Fig. 1, *f* and *g*).

At day 60, intima of allograft coronary arteries were thickened (84.1 ± 18.7 %, 21 arteries from four allografts) in the recipients treated with FK506 alone, whereas the treatment with antisense ODN to cdk2 kinase prevented arterial intimal thickening at day 60 (38.5 ± 19.8%, 18 arteries from three allografts, $P < 0.05$ vs. untransfected allografts at day 60). The effect of antisense cdk2 kinase ODN against intimal thickening was weakened at day 60; retransfection of ODN may be needed for the sustained inhibition of neointimal formation.

In this report, we clearly demonstrate for the first time that a single intraluminal administration of cdk2 kinase antisense ODN results in ODN stability within the vessel wall and prevents arterial neointimal formation. Intraluminal delivery of ODN during the interval between donation and implantation is clinically feasible; therefore, graft arteriopathy after transplantation is potentially a good application of this gene therapy. In addition, the HVJ–liposome method markedly enhances the therapeutic efficiency of ODN by increasing uptake, nuclear localization, and intracellular stability in the transplanted grafts. Further studies should be conducted in larger animal models to explore the clinical utility of this new technology for the long-term prevention of graft arteriosclerosis in humans.

Methods

Gene transfer to heart grafts. FITC-labeled phosphorothioate ODN was provided by Greiner Japan (Tokyo, Japan). FITC was labeled on the 3' and 5' ends of the ODN (16 bp) using fluorescein-ODN phosphoramidite. Transfer of phosphorothioate ODN was performed using the following protocol: HVJ complex with FITC-labeled phosphorothioate ODN (3 μM) was injected (0.2 ml) from the descending aorta of donor hearts which were then incubated for 10 min on ice. The grafts were transplanted immediately, harvested at 14 or 30 days after transplantation, and stored in optimum cutting temperature (OCT) compound. Sections were cut (6–8 μm), stained with eriochrome black T (Sigma Chemical Co.) solution, and examined by fluorescence microscopy.

Gene transfer complex and delivery. The sequences of ODN against human cdk2 kinase used in this study were antisense, 5'–GAA-GTT-CTC-CAT-GAA-GCG–3'; and sense, 5'–CGC-TTC-ATG-GAG-AAC-TTC–3' (–6 to +12 of human sequence; these sequences do not differ for mouse and human cdk2 kinase). Phosphatidylserine, phosphatidylcholine and cholesterol were mixed in a weight ratio of 1:4.8:2. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS) containing scrambled, sense or antisense ODNs. Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm² per s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (10,000 hemagglutinating units) in a total volume of 4 ml BSS. The mixture was incubated at 4 °C for 5 min and then for 30 min at 37 °C with gentle shaking. Free HVJ was removed from the HVJ–liposomes by sucrose density gradient centrifugation[11]. The liposomes were unilamellar, and the size of the HVJ–liposome complex containing ODN was 400–500 nm in diameter[14]. The phosphorothioate ODN (15 μM) was injected (0.2 ml) into the descending aorta of donor hearts just before transplantation.

Mice and treatment. Male BALB/c (H–2^d) and C3H/He (H–2^k) mice (age 4–6 weeks, 20–25 g) were obtained from Japan Charles River Laboratories (Tokyo, Japan). Donor BALB/c hearts were infused at the descending aorta with HVJ–liposome complex solution and incubated for 10 min on ice. The hearts were then immediately heterotopically transplanted into recipient C3H/He mice as described earlier[12,15]. All recipients were treated with FK506 (Fujisawa Pharmaceutical Co., Osaka, Japan) intraperitoneally at a dose of 0.1 mg/kg daily because C3H/He mice without any immunosuppressive treatment reject BALB/c allografts within 10 days[16]. *In vivo* gene transfer of antisense cdk2 kinase ODN ($n = 9$), sense cdk2 kinase ODN ($n = 7$), and scrambled ODN ($n = 6$) was performed using the HVJ–liposome method, and the allografts were harvested at day 30. Six mice received FK506 alone, and the allografts were harvested at day 30. For long-term observation, antisense cdk2 kinase ODN transfection ($n = 3$) and controls (no ODN transfection, $n = 4$) were performed; the allografts were harvested at day 60.

Tissue sections. Serial sections (6–8 μm) from tissue frozen in OCT compound were stained with Elastica van Gieson (EvG) to show the internal elastic lamina. The percentage of the lumen occluded by intimal thickening was calculated by two independent observers. The sections were photographed, blindly videodigitized and stored in an image analysis system (NIH Image). The area encompassed by the lumen and internal elastic lamina (IEL) was traced carefully and the cross-sectional area luminal stenosis was calculated by the formula: luminal occlusion = (IEL area – luminal area)/IEL area[17]. For immunohistochemical analysis, monoclonal antibodies to VCAM-1 (MK/2, American Type Culture Collection, Rockville, MD) and rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) were used. *In situ* detection of PCR-amplified murine cdk2 kinase mRNA was done according to the methods of Nuovo *et al*[14]. The cdk2 kinase 5' primer (nucleotides –6 to +12 of human sequences) was 5'–CGC-TTC-ATG-GAG-AAC-TTC–3'; the 3' primer (nucleotides 340–357) was 5'–ATG-GCA-GAA-AGC-TAG-GCC–3' (ref. 11). Reverse transcription solution containing antisense cdk2 kinase ODN primer was applied and incubated at 42 °C for 1 h. Then PCR solution was applied to the tissue sections and amplification done using the following parameters: 94 °C for 1 min; 55 °C for 2 min; 72 °C for 1 min; 30 cycles. The digoxigenin-labeled cDNA segments were detected by enzyme-linked immunoassay using anti-digoxigenin-AP, Fab fragments (Boehringer Mannheim, Germany). An enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (Boehringer Mannheim) produced an insoluble blue.

Statistical analysis. All pathological data are expressed as means ± s.d. Differences were compared using the Student's *t*-test. A two-sided value of $P < 0.05$ was considered statistically significant.

Acknowledgments

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RECEIVED 14 JANUARY; ACCEPTED 5 JUNE 1997

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Fusigenic viral liposome for gene therapy in cardiovascular diseases

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ABSTRACT **To improve the efficiency of liposome-mediated DNA transfer as a tool for gene therapy, we have developed a fusigenic liposome vector based on principles of viral cell fusion. The fusion proteins of hemagglutinating virus of Japan (HVJ; also Sendai virus) are complexed with liposomes that encapsulate oligodeoxynucleotide or plasmid DNA. Subsequent fusion of HVJ–liposomes with plasma membranes introduces the DNA directly into the cytoplasm. In addition, a DNA-binding nuclear protein is incorporated into the HVJ–liposome particle to enhance plasmid transgene expression. The fusigenic viral liposome vector has proven to be efficient for the intracellular introduction of oligodeoxynucleotide, as well as intact genes up to 100 kbp, both *in vitro* and *in vivo*. Many animal tissues have been found to be suitable targets for fusigenic viral liposome DNA transfer. In the cardiovascular system, we have documented successful cytostatic gene therapy in models of vascular proliferative disease using antisense oligodeoxynucleotides against cell cycle genes, double-stranded oligodeoxynucleotides as "decoys" to trap the transcription factor E2F, and expression of a transgene encoding the constitutive endothelial cell form of nitric oxide synthase. Similar strategies are also effective for the genetic engineering of vein grafts and for the treatment of a mouse model of immune-mediated glomerular disease.**

Vector Development

Construction of Fusigenic Viral Liposome. Although human gene therapy trials have been initiated, the clinical efficacy of these therapies has not been clearly demonstrated (1). It has been suggested that the limited success of current gene therapy trials may result in part from inadequacies of the DNA delivery systems (1). Improvements of viral and nonviral vector systems for gene therapy are being pursued actively. The development of novel viral vectors, such as pseudotype retrovirus vector (2), adenoviral vector of low antigenicity (3), and adeno-associated virus) vector, has been reported. More recently, the lentivirus vector appears to be promising for transducing nondividing cells (4). Similarly, new lipid formulations designed to increase the efficiency of transfection are being developed (5). Other novel delivery systems include lipopolyamine-based gene delivery (6), targeted gene delivery systems (7), and devices for particle bombardment (8).

We have focused our efforts on the development of a fusigenic liposome that is a hybrid vector between viral and nonviral technologies (Fig. 1; ref. 9). Hemagglutinating virus of Japan (HVJ; also Sendai virus) is a paramyxovirus that is 300 nm in diameter and contains two distinct glycoproteins (hemagglutinating neuroaminidase and fusion protein) in its envelope, which are involved in cell fusion (10). This virus is capable of fusing with the cell membrane at neutral pH, and these fusion properties can therefore be exploited to facilitate the introduction of DNA directly into cell cytoplasm, avoiding lysosomal degradation. Hemagglutinating neuroaminidase is required for viral particle binding to receptors consisting of sialoglycoproteins or sialolipids; hemagglutinating neuroaminidase then catalyzes the removal of sugars by its neuroaminidase activity. Fusion protein interacts with the lipid bilayer of cell membranes to induce cell fusion. Fusion protein is produced in an inactive form (F0) and is activated by proteolytic cleavage to the fusion polypeptides F1 and F2, which are held together by a disulfide bridge. The hydrophobic region of F1 can interact with cholesterol to induce cell fusion. Although liposomes themselves have no receptors for the virus, a direct interaction of F1 polypeptide with lipid is likely to play an important role in the ability of HVJ particles to fuse with liposomes (11). Several attempts have been made to incorporate DNA into the HVJ envelope itself (12) or into fusion products of HVJ with red blood cell ghosts (13), but these approaches were plagued by low trapping efficiency and/or low transduction efficiency. Since DNA can be efficiently encapsulated into liposomes (14), we turned to incorporation of HVJ envelope proteins into these liposomes. We first encapsulated DNA into liposomes consisting of phosphatidylcholine and cholesterol that were prepared via vortexing or reverse-phase evaporation. The trapping efficiency of DNA into such liposomes is 10–30%, so that 400–600 molecules of plasmid DNA and more than half million copies of 20-mer oligonucleotides were enclosed into one liposome particle. We then fused the liposomes with UV-inactivated HVJ to form fusigenic viral liposomes containing DNA (400–500 nm in diameter). HVJ–liposomes can fuse with plasma membranes, and fusion is completed within 10–30 min at 37°C. This short HVJ–liposome incubation time is particularly suited for *in vivo* gene therapy. In contrast, gene transfer by cationic liposomes generally requires a much longer incubation time of 5–20 hr. Indeed, we have shown that exposure of rat carotid artery to HVJ–liposomes containing fluorescein isothiocianate-labeled oligodeoxynucleotides (ODNs) for 10 min results in the uptake of fluorescence by 30–50% of cells within the vessel wall. Other advantages of HVJ–liposome-mediated delivery are the introduction of molecules directly into the cytoplasm and avoidance of degradation in the endosome and lysosome. In fact, when fluorescein isothiocianate-ODN was introduced into vascular smooth muscle cells (VSMCs) using HVJ–liposomes, fluorescence was detected in cell nuclei within 5 min, and the fluorescence remained prominent in the nuclei for at least 72

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Abbreviations: HVJ, hemagglutinating virus of Japan; ODN, oligodeoxynucleotide; VSMC, vascular smooth muscle cell; AS, antisense; PCNA, proliferating cell nuclear antigen; cdc2, cell division cycle 2; ec-NOS, endothelial cell NO synthase.

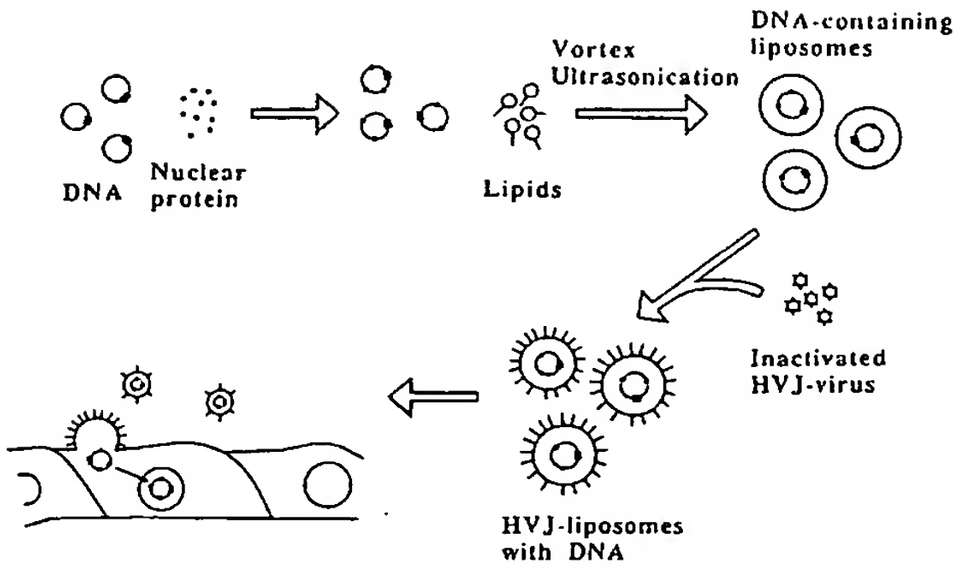

FIG. 1. Procedure of gene transfer by HVJ–liposome.

hr (15). In contrast, after direct transfer of fluorescein isothiocianate-ODN to VSMCs without the HVJ lipsome, fluorescence was observed in cytoplasmic compartments but not in the nucleus. Furthermore, the fluorescence was no longer detectable at 24 hr after direct fluorescein isothiocianate-ODN application.

Current gene transfer methods are also limited by the low level of expression of the transgene. We have found that cointroduction of plasmid DNA with a nuclear protein, high mobility group-1, can enhance transgene expression in animal tissues (16, 17). High mobility group-1 protein is a nonhistone DNA-binding protein of 28 kDa. It is reported that high mobility group-1 is required for the bending or looping of DNA and for enhancing transcription by specific recognition of cruciform DNA (18). An advantage of the HVJ–liposome is the capacity for such cointroduction of both DNA and proteins via their incorporation into the same particle. Indeed, we have recently cointroduced RNase H with antisense (AS) ODN for angiotensin converting enzyme *in vivo* into injured rat carotid artery. We observed that the AS effect was augmented 3-fold with the addition of RNase H (unpublished data). Thus, HVJ–liposomes have been useful for DNA transfer in various tissues *in vivo*, resulting in functional gene expression or gene suppression (Table 1).

Advantages of Fusigenic Viral Liposome. *Efficient transfection of oligonucleotides, plasmid DNA, and proteins.* The HVJ–liposome can encapsulate DNA up to 100 kbp. We have succeeded in transducing cosmid DNA (45 kbp) containing the thymidine kinase gene into cultured mouse cells (19). Recently, full-length cDNA of human Ducchene muscular dystrophy gene was introduced *in vivo* using HVJ–liposomes, resulting in its expression in skeletal muscle and diaphragm of the mdx mouse (20). When AS ODN to basic fibroblast growth factor was transfected into VSMCs using HVJ–liposomes and compared with cationic lipid transfection or direct ODN transfer without any vector, the concentration of AS basic fibroblast growth factor required to reduce cellular DNA synthesis by 75% was approximately 0.1 μM, 10 μM, and 20 μM, respectively (21). Thus, the HVJ–liposome is an effective method for ODN and plasmid DNA transfer. Ribozymes have also been efficiently introduced into cells using HVJ–liposomes, and the vector has also been useful for the introduction of recombinant proteins IgG and IgM (ref. 20 and unpublished data).

Penetration of the vector into tissues in vivo. Efficient *in vivo* transfer and expression of transgenes have been observed in cells of the tunica media of intact rabbit carotid arteries after filling the lumen with HVJ–liposomes containing the trans-

Table 1. *In vivo* gene transfer by HVJ–liposome

Organ	Gene product	Duration of gene expression
Liver		
Rat and mouse	Insulin	7–14 days
Rat	Renin, HBsAg	7 days
Rat	LacZ	>4 weeks
Kidney		
Rat	TGF-β, PDGF	7 days
	SV40—large tag	
Heart		
Rat	TGF-β, HSP70,	>2 weeks
	Mn-SOD, Bcl-2	>2 weeks
Skeletal muscle		
Mouse	Dystrophin	2 weeks
Rat	Luciferase	>4 weeks
Rat	Decorin	>2 weeks
Artery		
Rat	SV40, ACE, c-NOS, p21, ANP	>2 weeks
Rabbit	p53	>2 weeks
Lung		
Rat	TGF-β, PDGF, LacZ	>2 weeks
Patellar ligament		
Rat	LacZ	>4 weeks
Brain		
Rat	LacZ	>2 weeks
Eye		
Mouse and Monkey	LacZ	>2 weeks
Skin		
Rat	LacZ	7–10 days
Testis		
Mouse	CAT	>8 months

HBsAg, hepatitis B surface antigen; TGF-β, transforming growth factor β; PDGF, platelet-derived growth factor; SV40, simian virus 40; HSP70, heat shock proteins 70; SOD, superoxide dismutase; ACE, angiotensin converting enzyme; c-NOS, constitutive NO synthase; ANP, atrial natriuretic protein; and CAT, chloramphenicol acetyltransferase.

gene for 10 min under 150 mmHg (1 mmHg = 133 Pa). Thus, in contrast to adenoviral vectors, this vector appears to penetrate readily the intimal layer to reach the tunica media. Our observation that the interstitium of the tunica media is stained upon incubation with liposomes without HVJ containing Evans blue dye suggests that this penetrating ability is conferred primarily by the liposome. Subsequent cell fusion and intracellular delivery of DNA is mediated by HVJ fusion proteins. Our liposome consists of the negatively charged phosphatidylserine, in addition to phosphatidylcholine and cholesterol. The presence of this negative charge may play an important role in enhancing transmigration into the vessel wall, and we are currently varying the composition of the liposome to test its effect on tissue penetration. It is our observation that negatively charged liposomes generally do not work well for DNA transfer into cultured cells *in vitro*. However, the converse may be true of *in vivo* gene transfer. Recently, we developed cationic HVJ–liposomes, and compared their gene transfer efficacy with those for anionic HVJ–liposomes and cationic lipids (Lipofectamine; GIBCO/BRL) *in vitro* and *in vivo*. Cationic HVJ–liposomes and Lipofectamine are much more efficient than anionic HVJ–liposomes for achieving luciferase gene expression *in vitro*. In contrast, negatively charged HVJ–liposomes are most efficient for *in vivo* transfection of liver and skeletal muscle.

No apparent toxicity and low antigenicity. Thus far, using HVJ–liposomes, we have not observed significant cell damage *in vitro*, nor have we detected target organ dysfunction *in vivo*. Up to 10^{10}-10^{11} HVJ lipsome particles have been injected *in vivo* into the portal veins of 8-week-old mice without any detectable toxicity (22). However, the fate of the HVJ proteins and the virion, as well as that of the lipids, must be analyzed more precisely before the application of HVJ–liposomes for human clinical trial. Furthermore, the effectiveness of UV light for the complete inactivation of HVJ must be documented carefully.

We have also examined the antigenicity of HVJ–liposomes *in vivo*. Low titers of antibodies against HVJ could be detected 1 week after injection of the HVJ–liposome into the portal vein of the rat. When HVJ–liposomes containing marker genes were injected into the portal veins of rats that had received a prior injection of empty HVJ–liposomes 7 days earlier, the marker gene expression was not attenuated, compared with rats undergoing primary HVJ–liposome transfection. Clearly, much more work has to be done to study the immunogenicity of the HVJ–liposome complex and to define the effect of repeated injections *in vivo*.

Improvement of current vector system. The transient nature of gene expression is a major limitation of the current HVJ–liposome system. Recently, we have succeeded in achieving longer term gene expression *in vivo* using the self-replicating apparatus of Epstein–Barr virus (ref. 22 and unpublished data). A plasmid containing the Ori P sequence and the EBNA-1 coding region derived from Epstein–Barr virus was constructed, and the luciferase gene, expressed under the control of chicken β-actin promotor, was cloned into this vector. Luciferase gene expression in cultured human cells (HeLa and KEK-293) increased with cell division after HVJ–liposome transfection with this vector. Southern blot analysis of episomal DNA in these cells indicated that the transgene replicated autonomously in the nucleus. However, this plasmid could not replicate autonomously in rodent cells but was retained in the nucleus. When this Epstein–Barr virus replicon vector was introduced into rat liver using HVJ–liposomes, luciferase gene expression was detected for >4 weeks, although the level gradually decreased. To enhance tissue-specific expression, the transgenes encapsulated into HVJ–liposomes have now been designed to be driven by cell type-specific promoters. We have succeeded in achieving gene expression in the liver by the use of the mouse albumin promotor or the rat pyruvate kinase promotor, and the endothelin promotor may also allow endothelial cell specific transgene expression *in vivo*.

Application of Fusigenic Virus Liposome to Gene Therapy of Cardiovascular Diseases

Vascular Proliferative Disease (e.g., Restenosis). *AS strategy.* Balloon angioplasty is one of the major therapeutic approaches to coronary artery stenosis. Restenosis, however, occurs in 30–40% of patients after angioplasty. A major component of restenosis is neointimal hyperplasia, which is characterized primarily by abnormal growth and migration of VSMCs. Multiple growth factors are involved in the stimulation of VSMC growth. Cell cycle progression to cell division is ultimately regulated by cell cycle regulatory genes. We have therefore developed a strategy to inhibit abnormal growth of VSMC *in vivo* by suppressing the expression of cell cycle regulatory proteins. Indeed, we reported that the combination of AS ODN against proliferating cell nuclear antigen (PCNA) and cell division cycle 2 (cdc2) kinase inhibited serum-stimulated VSMC proliferation *in vitro* (23, 24). Similarly, the combinations of AS cdc2 kinase/AS cyclin B1 and AS cdc2 kinase/AS cyclin-dependent kinase 2 completely inhibited serum-stimulated DNA synthesis. Since neointima formation is initiated by an acute phase of medial smooth muscle cell replication, we transfected AS ODN to PCNA and cdc2 kinase via HVJ–liposomes into balloon-injured rat carotid arteries *in vivo*. As shown in Fig. 2, neointima formation was completely inhibited for 2 weeks after AS ODN transfer, and the inhibitory effect was sustained up to 8 weeks after a single transfection. However, no inhibitory effect was observed after transfection with control sense ODN. Combinations of AS ODN with cdc2 kinase/cyclin B1 and AS with cdc2 kinase/cyclin-dependent kinase 2 also resulted in suppression of neointimal hyperplasia in this experimental model of vascular proliferative disease.

Transcriptional factor decoy strategy. The transcriptions of PCNA, cdc2 kinase, and c-*myc* and c-*myb* protooncogenes are activated by a common transcriptional factor, E2F. In quiescent VSMCs, E2F forms a protein complex with retinoblastoma gene product, RB. Upon growth stimulation, the RB protein is phosphorylated, and E2F is subsequently released

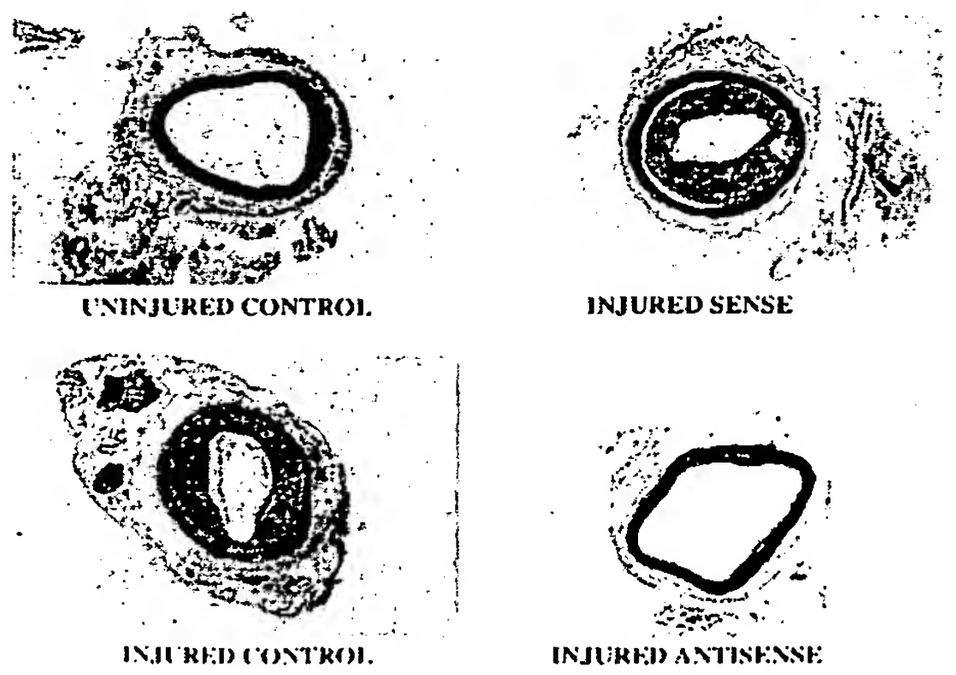

FIG. 2. Long-term suppression of neointima formation by AS-cdc2 kinase and AS-PCNA. Uninjured rat carotid artery (*Upper Left*), injured rat carotid artery without protein–liposome (*Lower Left*), injured rat carotid artery treated with protein–liposome containing 15 μM sense ODNs for both molecules (*Upper Right*), and injured rat carotid artery treated with protein–liposome containing 15 μM AS ODNs (*Lower Right*) were shown. At 2 weeks after transfection, rats were killed and vessels were fixed with 4% paraformalehyde.

from the complex. E2F then binds to the promoter region of the above cell cycle genes and activates their transcription. The consensus sequence TTTCGCGC is the binding site for E2F. Our strategy for inhibition of cell proliferation is the intracellular delivery of double-stranded ODN containing the TTTCGCGC sequence to act as a decoy to trap the released E2F (25). We synthesized a 14-mer as well as a 30-mer double-stranded ODN containing the consensus sequence and demonstrated that both are effective E2F based on competitive gel-shift assay. Using HVJ–liposomes, E2F decoy ODN was then introduced into cultured VSMCs, and it completely inhibited serum-stimulated growth. This growth inhibition was accompanied by reductions in PCNA and cdc2 kinase levels in these VSMCs. In contrast, mismatched decoy showed no inhibitory effect.

Based on these *in vitro* results, we examined the effect of E2F decoy on the prevention of neointimal hyperplasia *in vivo*. E2F decoy was transduced into balloon-injured rat carotid arteries using HVJ–liposomes. Our results demonstrated a marked suppression of neointimal formation at 2 weeks after balloon injury. In contrast, mismatched, scrambled, or progesterone responsive element decoy had no effect on neointimal development. Interestingly, we observed that a single administration of E2F decoy resulted in a sustained inhibition of neointimal formation up to 8 weeks after the treatment.

Gene transfer approach. Using HVJ–liposomes, we also attempted to inhibit neointimal formation by plasmid DNA gene transfer (26). Several studies had suggested NO could inhibit neointimal formation. For example, NO inhibited VSMC growth and migration *in vitro*. Systemic administration of a NO synthase inhibitor accelerated atherosclerotic lesion formation and impaired vascular reactivity. We therefore postulated that overexpression of endothelial cell NO synthase (ec-NOS) is an effective gene therapeutic strategy. Accordingly, we transfected balloon-injured rat carotid arteries with an expression vector containing the ec-NOS gene.

Four days after HVJ–liposome-mediated ec-NOS gene transfer into injured rat carotid arteries, significant levels of ec-NOS protein expression were detected. Consequently, NO production in the injured artery was enhanced by ec-NOS gene transfer. Two weeks after ec-NOS gene transfer, histological analysis revealed a 70% reduction in neointimal area as compared with the nontransfected injured artery (26). In contrast, no inhibition of neointima formation was observed in injured vessels undergoing control vector transfection. Since NO has multiple effects on the vessel wall, including vasorelaxation, inhibition of platelet aggregation, prevention of leukocyte adhesion, and suppression of VSMC growth and migration, we propose that our strategy to augment NO production may be an effective and practical approach to the gene therapy of restenosis.

Another important consideration for the therapy of restenosis is reendothelialization of the injured artery. Although several factors are known to stimulate endothelial cell growth, we have recently found that hepatocyte growth factor is a more potent accelerator of endothelialization than either vascular endothelial cell growth factor or basic fibroblast growth factor. In addition, unlike basic fibroblast growth factor, hepatocyte growth factor does not stimulate VSMC growth. We are therefore developing a strategy to prevent restenosis via the inhibition of VSMC growth using an AS, decoy, or NOS gene transfer approach in combination with the stimulation of endothelial cell growth by hepatocyte growth factor gene transfer.

Genetic engineering of vein grafts resistant to atherosclerosis. Saphenous vein grafts are the most commonly used bypass conduits for the treatment of occlusive vascular disease. However, up to 50% of vein grafts fail within a period of 10 years, primarily as a result of accelerated graft atherosclerosis. When grafted into arteries, veins are subjected to increased intraluminal pressure and undergo adaptive wall thickening. This thickening, however, involves neointimal hyperplasia, and this neointimal layer is believed to form the substrate for the aggressive atherosclerotic disease that eventually causes graft failure. We therefore hypothesized that a cytostatic strategy to prevent the hyperplastic response to the acute injury of grafting would redirect the biology of vein graft adaptation away from neointimal hyperplasia and toward medial hypertrophy (27). Rabbit jugular vein was isolated and transfected with AS ODN against PCNA and cdc2 kinase using HVJ–liposomes. The transfected vein was then grafted into the carotid artery. Neointima formation inhibited in the AS ODN-treated vein grafts for up to 10 weeks after surgery. In response to cell-cycle arrest with AS ODN, the genetically engineered vein grafts developed hypertrophy of the medial layer. When the rabbits were fed a high-cholesterol diet, accelerated atherosclerotic changes, characterized by plaque formation and macrophage infiltration, developed in the untreated and control ODN-treated grafts. In contrast, neither plaque formation nor significant macrophage infiltration was observed in any of the AS ODN-treated grafts, despite cholesterol feeding. These results establish the feasibility of developing genetically engineered bioprostheses that are resistant to failure and better suited to the long-term treatment of occlusive vascular diseases.

Treatment of glomerulosclerosis. We have also used E2F decoy oligonucleotide to ameliorate the changes seen in an animal model of mesangial proliferative nephritis. Injection of anti-Thy-1 antibody, which specifically injures glomerular mesangial cells, results in a proliferative glomerular lesion. We demonstrated that intrarenal arterial perfusion of HVJ–liposome complexes containing 14-mer E2F double-stranded decoy ODN inhibited anti-Thy-1-induced mesangial cells proliferation, as documented by BrdUrd incorporation and total glomerular cell counts. Furthermore, this decoy treatment prevented histopathologic changes in the glomeruli that closely mimic the mesangioproliferative nephritis seen in IgA nephropathy and in some forms of focal glomerular sclerosis.

Future Direction

The fusigenic viral liposome appears to be an effective tool for gene transfer and therapy. Our current system is an HVJ–liposome complex, but other viral fusion proteins may be applicable. In addition, in forming fusigenic liposome complexes, purified or recombinant fusion polypeptides may be used instead of the entire viral envelope. Since the system is a hybrid between viral and nonviral vectors, safety issues must be considered. It will be necessary to test the safety of UV-inactivated HVJ itself, as well as the safety of the liposome and the immunogenicity of the HVJ–liposome complex. HVJ–liposomes may be useful for short-term and local gene therapy. Modifications of this system will be necessary to permit high levels of stable expression of the transgene for clinical therapy.

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Reference 3

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Clinical review

Science, medicine, and the future: Molecular genetic approaches to understanding disease

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Abstract

Molecular genetics has greatly increased the understanding of diseases in which there is a single gene defect such as cystic fibrosis. Discovering the gene responsible and its function not only helps determine the pathogenesis of the disease but also offers a possible treatment–gene therapy. Polygenic disorders such as diabetes may soon yield their secrets to the same approach. Animal models of genetic diseases are proving useful research tools, and transgenesis has made xenografting possible. Furthermore, antisense technology allows specific inhibition of undesirably overexpressed genes such as those driving unwanted vascular cell proliferation and restenosis after angioplasty. The completion of the human genome project should make the search for "disease" genes much quicker and will increase still further the importance of these gene based approaches toward diseases.

Introduction

If you think that splicing is a carpentry technique or that a knockout is the end of a politically incorrect sporting contest you should read on. Indeed, even if you are one of the many doctors who knows that it is now routine to identify genes

and manipulate their expression, the hazier details may be clarified by this article. My aim is to set out important genetic approaches toward understanding disease and introduce how advances in biomedical science may affect medicine in the next 15 years or so. Genetic terms are explained in the glossary. In my next article I will cover cellular approaches. The objective of subsequent articles in this series will be to foresee how modern science will impinge on the prevention, diagnosis, and treatment of common disease processes such as coronary heart disease, hypertension, and lung cancer.

Molecular genetics

Modern molecular techniques have revolutionised attempts to understand the pathogenesis of single gene disorders such as cystic fibrosis. The discovery of abnormal alleles seems almost routine, and the potential rewards of "finding the gene" are great. In cystic fibrosis the transmembrane conductance regulator (CFTR) is inactivated by various mutations. Discovering the gene gave an insight into pathogenesis–it was quickly realised that CFTR is an epithelial cell surface chloride transporter, which explains known defects in ion transport across epithelia in patients with cystic fibrosis. Secondly, there was a ready made candidate treatment–transfer to diseased tissue of the complementary DNA (cDNA) of the normal CFTR gene which can then be used by the cells to produce normal protein (see below) and reconstitute chloride transport.

Molecular geneticists have made remarkable contributions to the understanding of single gene disorders. Some studies have taken the candidate gene approach–scientists make an educated guess that a well characterised allele lies at or close to a disease locus. More usually, investigators undertake painstaking linkage analysis of DNA specimens from affected families. They seek to associate carefully documented cases of disease with particular chromosomal "landmarks" and then aim to find the gene in a relatively short segment of DNA. This positional cloning approach has been greatly facilitated by a comprehensive map of microsatellites–regions of DNA that vary greatly in their sequence between individuals but which occupy identical chromosomal positions.

Once a gene is mapped to a particular section of DNA various strategies can be used to speed the hunt for the gene among the millions of nucleotide bases in non-coding "junk" DNA, which constitutes up to 90% of the human genome. For example, help may come from the growing map of expressed sequence tags, a library of partially sequenced cDNAs obtained from unselected mRNAs. Furthermore, large chunks of chromosomal DNA can be handily stored,

manipulated, and screened by being incorporated into a library of yeast artificial chromosomes that contain overlapping segments of human DNA–so called contigs (fig 1). However, positional cloning may still be difficult. It took years to find *PKD1*, the gene responsible for most cases of autosomal dominant polycystic kidney disease, because it was hidden in a cluster of similar genes on chromosome 16. Such difficulties may be removed by the mapping of the human genome.

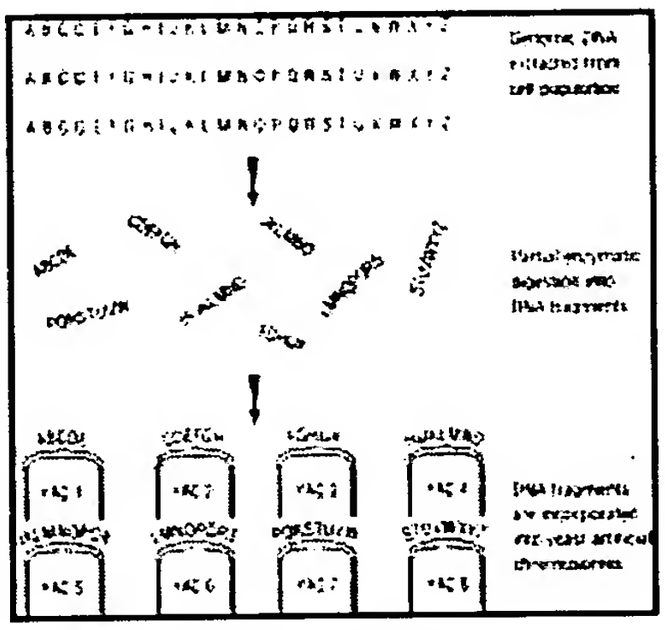

Fig 1 Assembling a contig of genomic DNA in a yeast artificial chromosome (YAC) library. Genes which map close to marker H may be found in YACs 2, 3, or 4

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Bolstered by success with uncommon single gene disorders, molecular geneticists are turning to major causes of ill health and premature death such as coronary heart disease, hypertension, and diabetes mellitus. These disorders cluster in families but exhibit polygenic inheritance, expression of the disease phenotype apparently depending on the interaction of several genes, which may in turn interact with environmental factors. Genome screening is now feasible thanks to automated techniques and microsatellite markers, but such linkage studies require careful interpretation. One aim of linkage studies is to help determine pathogenesis by identifying gene products involved in disease. However, we will need a strong ethical framework with which to handle the knowledge that a particular gene is associated with increased risk of a serious disorder. Telling someone glibly that they have a fivefold increased risk of early death from coronary heart disease may not be ideal.

Gene transfer and gene therapy

Gene transfer is a simple and attractive concept (fig 2). All you need to do is take a piece of DNA that encodes for the desired protein and transfer it to a cell. The cell then transcribes the gene and translates the resulting mRNA to generate

protein. It is easy to appreciate why gene transfer is causing so much excitement. Firstly, transferring human genes to simple organisms or cell lines allows production of large amounts of "therapeutic" proteins such as erythropoietin. Secondly, there is enthusiasm for using gene transfer to patients to treat disease–so called gene therapy. For example, it is hoped that normal epithelial function and defence against infection may be restored in the airways of patients with cystic fibrosis by transferring the normal CFTR gene.

Conclusions

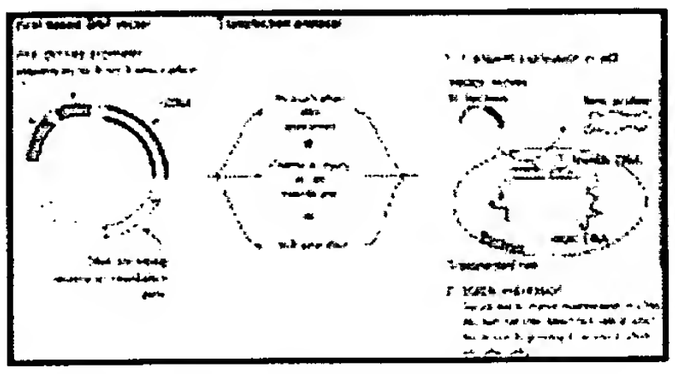

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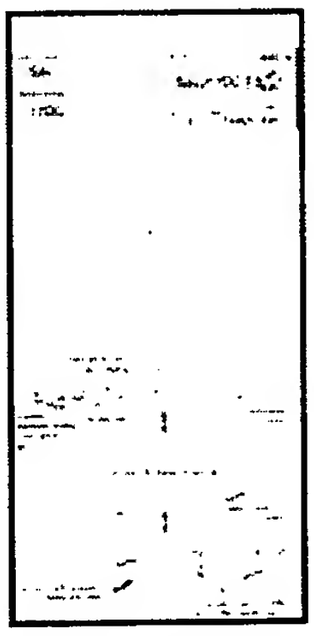
Fig 2 Principles of gene transfer. cDNA for a protein foreign to the host cell is incorporated into a DNA vector, which is then introduced or "transfected" into cells. The cDNA will be transiently expressed before being eliminated. However, occasionally vector DNA incorporates into cellular DNA, and these stably transfected cells can be selected in experimental settings

However, behind the simple concept lies a large and incomplete body of knowledge. A functioning gene is not merely composed of the encoding DNA sequence (fig 3). Upstream of the site at which gene transcription is initiated there is a promoter region of DNA sequences that bind nuclear proteins called transcription factors. These factors regulate the initiation of transcription. Indeed successful elongation and processing of a gene transcript may require coordinated action of many pieces of genomic DNA which are not expressed in the final mRNA transcript. Therefore, although a cDNA can be "read" to generate a full length mRNA and protein, gene transfer will not work unless the cDNA is combined with elements designed to drive transcription. These are usually provided by incorporating the cDNA into a vector–a piece of DNA which is derived from a virus and which bears powerful promoter sequences which will bind host cell transcription factors and allow high level expression of the transferred cDNA.

Fig 3 Control of gene expression. Transcription of DNA by a multiprotein complex with RNA polymerase activity is regulated by binding transcription factor proteins just upstream (or 5') of the open reading frame of the gene. RNA arising from introns is spliced out to yield mature mRNA which arises from the exons of the gene by means of the "cut and paste" process shown. The capacity of mRNA to be translated by ribosomes can be regulated by RNA sequences in the 3' untranslated region downstream of the trinucleotide stop codon which halts translation



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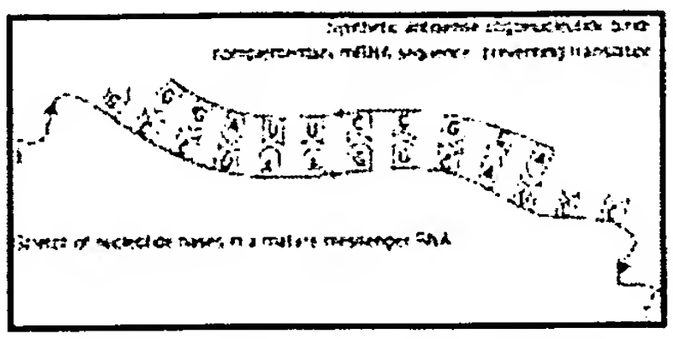
Both the art and science of gene transfer revolve around which vectors to use and how to get them into the cells of interest. In gene therapy it is important that the vector must not cause adverse effects such as immune or inflammatory reactions, expression in the wrong tissue, or, worse, spread to other people.

Inhibiting gene expression may also be therapeutic

Techniques designed to transfer cDNA constructs into cells can also be exploited to interfere with gene expression by administering antisense oligonucleotides (fig 4). These consist of 20 or so nucleotides synthesised in a sequence which complements the mRNA of interest. The oligonucleotides bind the mRNA and prevent translation of the protein. Antisense oligonucleotides can be administered in vivo and have great promise in treating disorders of cell proliferation. For example, the fibrocellular intimal hyperplasia underlying restenosis of atheromatous vessels after successful angioplasty can be blocked by antisense to the gene *c-myb*, which encodes a nuclear protein essential for cell division. However, because antisense strategies don't always work there is growing interest in using ribozymes, enzymes which specifically recognise and degrade particular mRNAs. Although antisense strategies might lack the glamour of gene therapy, the ability selectively to turn off gene expression may prove particularly important in medicine.

Fig 4 An antisense oligonucleotide prevents translation of an m

RNA



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Knockout and transgenic technologies

Perhaps the best test of a gene function is provided by manipulating the genome of experimental animals. This is easiest in mice because scientists can now culture undifferentiated but pluripotential murine embryonic stem cells. Genetically altered embryonic stem cells can be microinjected into early embryos so that the manipulated cells contribute to all cell types of the resulting chimera, including germ line cells. Breeding these "founder" mice produces individuals carrying one copy of the altered gene and one normal copy (heterozygotes) or mice carrying two altered copies (homozygotes).

This technique has proved particularly useful in generating models of disease in which there is a genetic "loss of function." So called knockout mice are created by means of homologous recombination and targeted mutagenesis, techniques by which the gene of interest is inactivated by swapping the normal DNA for a similar DNA construct with a mutation. The DNA "plugged in" to the embryonic stem cell genome also contains a selection element such as a neomycin resistance gene so that cells bearing the desired mutation can be selected prior to injection into embryos because the knockout cells can propagate in otherwise toxic neomycin.

Knockout mice can also be used to model the consequences of somatic mutations which arise after birth and are believed to be particularly important in carcinogenesis. Mice deficient in the tumour suppressor gene *p53* in the germ line are models of human Li-Fraumeni syndrome, developing multiple tumours because of defects in deletion of cells in which ionising radiation causes somatic mutations which activate tumour promoting genes. Indeed, somatic mutations inactivating *p53* contribute to the pathogenesis of about a third of tumours. It will soon be possible to model such mutations by exploiting new techniques that allow you to switch on gene inactivation in particular cell lineages at will.

Transgenic technology, in which a normal gene is overexpressed for investigative

purposes, preceded embryonic stem cell techniques and can be used in animals other than mice. However, transgenesis without embryonic stem cells is much more hit and miss because it relies on chance incorporation of DNA injected into early embryos. Nevertheless, the technique works, and the most dramatic practical application of transgenesis may prove to be in xenografts–transplants from animals to man. Normally, pig kidneys are rejected rapidly by primates because the recipient has high titres of antibodies which react with the glycoproteins on the surface of pig cells, especially endothelial cells lining the vasculature of the graft. Antibody fixation leads to complement activation, endothelial injury, thrombosis, and graft loss–so called hyperacute rejection. However, these phenomena are suppressed if the transplanted pig tissue overexpresses human cell surface proteins that regulate the activation of the complement system. Therefore pigs transgenic for complement regulatory proteins could be used as a source of organs for human transplantation. Nevertheless, apart from ethical and safety considerations, xenografts may prove susceptible to long term immunological injury by T cells rather than complement.

Molecular microbiology

Lastly, genetic approaches are proving important in infectious and parasitic diseases. By applying modern molecular genetic techniques to the study of microbial pathogenicity and to host resistance susceptibility, new insights are being gained into treatment and prevention, by vaccination and other strategies. For example, the particularly challenging problem of severe malaria due to *Plasmodium falciparum*, which resists both treatment and attempts at prevention by vaccination, is being addressed by a two pronged genetic approach. Firstly, a yeast artificial chromosome and expressed sequence tag map (see above) of the *P falciparum* genome is being constructed to speed up molecular analyses of potential targets in the parasite for treatment and vaccines. Secondly, attempts are being made to identify human genes which determine deleterious or advantageous responses to infection. Together these approaches could yield new vaccine candidates and new treatments.

Glossary

Allele–One of two or more alternative forms of a gene which occupy the same locus (position) on a particular chromosome. Homozygotes bear identical alleles at two corresponding loci on a pair of chromosomes; heterozygotes have two different alleles.

Complementary DNA (cDNA)–A DNA copy made from a messenger RNA template which is transcribed in a "finish to start" orientation by a reverse

transcriptase enzyme. When the cDNA is transcribed in the conventional orientation by an ordinary RNA polymerase enzyme a copy of the parent mRNA is produced. Consequently, cDNAs are valuable when one wants to mimic endogenous expression of a gene.

DNA construct—An engineered piece of DNA, usually designed for incorporation into a viral based vector for expression in other cells. For example, a construct might incorporate a deliberately mutated cDNA such that an abnormal form of the corresponding protein is ultimately produced.

Homologous recombination—Exchange of chromosomal DNA, by recombination or "crossing over" for another stretch of DNA which is homologous or similar. However, in gene manipulation experiments the replacement DNA has been subtly altered to affect the properties of the transcribed/translated product.

Positional cloning—Aims to identify the locus and the gene responsible for a particular disorder/protein relative to mapped markers, usually microsatellites. This is determined by linkage analysis which assesses the frequency with which a particular marker cosegregates with the disorder/protein in families. Because there may be crossing over of DNA between a pair of chromosomes the further a gene is from a marker the lower the chance of cosegregation. Cloning involves sequencing the gene in its entirety, usually in overlapping segments.

Targeted mutagenesis—A powerful technique in which particular bases in DNA are deliberately mutated to alter the properties of the mRNA resulting from DNA transcription by RNA polymerase. In turn this can alter the amino acids in the encoded protein, truncate the protein, or prevent protein expression altogether.

Transcription factors—Proteins which bind "promoter" regions of DNA upstream of the sequences which encode mRNA, thereby leading to assembly of an RNA polymerase and transcription of the gene.

Yeast artificial chromosomes (YACs)—Can accommodate long stretches of foreign DNA and act as storage vessels which can be propagated in yeast cells in culture.

Conclusions

The potential power of genetic approaches to disease is already great. My next article will deal with the rapidly evolving discipline of molecular cell biology. The tools described above are being used to investigate many different disorders. Furthermore, the importance of genetic approaches may redouble with the anticipated success of the human genome project. Watch this space.

Rapid Responses:

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alarm over animal models

Alfred N Jackson

bmj.com, 10 Sep 2004 [Full text]

Reference 4

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(54) Title: USE OF FAS LIGAND TO SUPPRESS LYMPHOCYTE-MEDIATED IMMUNE RESPONSES

(57) Abstract

Soluble mouse and human Fas ligand polypeptides and methods for inhibiting T-lymphocyte-mediated immune responses, including those directed against autologous and/or heterologous tissues, e.g., by a recipient mammal of a transplanted tissue, by providing the recipient mammal with Fas ligand. The Fas ligand may be provided to the recipient mammal by a variety of means, including by direct administration of the Fas ligand or by providing the gene encoding the Fas ligand to a subject such that Fas ligand is synthesized by the subject.

「移植」 Vol31. 総会臨時号. (1996) P. 180

Reference. 5

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マウス同種膵島・睾丸片同時移植-FasLを介した免疫制御

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【目的】眼球や睾丸はFasLの発現が高く、活性化したT細胞をアポトーシスに陥らせるため、他の部位に比較して移植片が長期に生着することが示されている。このFasLを介した免疫機作が同所性に移植した移植片の拒絶を制御しうるものかどうかを、睾丸・膵島同時移植の系において検討した。【方法】膵島および睾丸片 (TG) のドナーとしてC3H、および機能的なFasLを欠如したC3H-*gld*をもちいた。レシピエントとしてC57BL/6、および機能的なFasを欠如したC57BL/6-*lpr*をもちいた。TGを腎被膜下に移植し、経時的に組織学的検討をおこなった。次にSTZにより糖尿病を誘発したC57BL/6、C57BL/6-*lpr* にC3Hの膵島と、C3H又はC3H-*gld*のTGを同時に腎被膜下に移植した。【結果】C3HのTGは細胞浸潤は少なく、3週目まで組織の生着が認められたが、C3H-*gld*では早期に拒絶された。C3Hの膵島のみを移植した群では平均生着日数は10±2日であったが、C3Hの膵島とC3HのTGを同時に移植した群では、16±5日と有意な生着延長効果が認められた ($p<0.01$)。一方、C3Hの膵島とC3H-*gld*のTGの移植群では11±3日と生着延長は認められなかった。また、C3Hの膵島・C3HのTGのC57BL/6-*lpr*への移植でも有意な生着延長は認められなかった。

【結語】膵島を睾丸片とともに移植することにより、膵島の拒絶反応が抑制され、その機作は移植片のFasLとレシピエントのFasを介した反応であることが推察された。

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移植免疫反応におけるFasリガンドの役割

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[目的] Immune-privileged site の一つとして精巣が知られている。これは精巣細胞に恒常的に発現されたFasリガンド(FasL)によって担われており、この性質の応用は移植拒絶反応の防御に有効であることを示唆する報告が昨年なされた。しかし我々はFasL遺伝子導入細胞を皮下に移植し、それとは逆に、FasL依存的な激しい拒絶反応が起こりうることを報告した(Nature 379, 1996)。今回、このFasL依存的拒絶反応のメカニズムについて検討した。[方法と結果] FasLを発現させたマウスlymphoma(FasL/L5178Y)とその親株細胞を同系マウスの皮下または腹腔に移植すると、親株細胞は腫瘤を形成し生着するが、FasL/L5178Yは早やかに拒絶され腫瘤の形成は見られなかった。この拒絶は胸腺依存性T細胞を欠失したヌードマウスに移植した場合にも観察されることから、CTLに非依存的であることが示唆された。抗体で顆粒球を除去したマウスではこの拒絶は見られず、顆粒球が重要な働きを担っていると考えられた。移植部位の組織の検討では、拒絶に関わるエフェクター細胞は好中球であることが示唆された。以上の結果から、FasLは何らかの機序で局所に炎症反応を引き起こし、移植細胞の拒絶に働くことが示唆された。[考察] 移植片上に発現されたFasLは、発現細胞又は移植部位の違いによって、拒絶抑制よりむしろ促進に働く可能性があると考えられた。アポトーシス誘導分子と考えられてきたFasLに、起炎性サイトカインとしての新たな機能が存在することが示唆された。

"Japanese Journal of Transplantation", extra edition featuring the general meeting of the Japan Society for Transplantation, Vol. 31, p. 180 (1996)

47 Co-transplantation of Murine Allogenic Islets of Langerhans and Testicular Grafts - FasL-mediated Immunosuppression

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[Object] It has been indicated that the eye and the testis highly express Fas ligand (FasL) to induce the apoptosis of activated T cells so that the grafts derived from such organs survive over a longer period of time as compared with other sites. We investigated on a system of co-transplantation of testes and pancreatic islets whether or not the FasL-mediated immune mechanism as above can

control the rejection of orthotopic grafts. [Method] C3H, and C3H-*gld* lacking functional FasL were used as the donor of islets and testis grafts (TGs). C57BL/6, and C57BL/6-*lpr* lacking functional FasL were used as the recipient. TGs were transplanted under the renal capsule and histological analysis was carried out with time. Then, islets from C3H were transplanted to the STZ-induced diabetic C57BL/6 or C57BL/6-*lpr* under the renal capsule thereof together with TGs from C3H or C3H-*gld*. [Result] The TGs from C3H were scarcely subjected to cellular infiltration and the tissues survived up to the third week after transplantation, whereas those from C3H-*gld* were rejected at an early stage. As for the average number of survival days of the islets from C3H, it was 10 ± 2 for the group of animals to which the islets only were transplanted while 16 ± 5 for the group to which the islets were transplanted together with the TGs from C3H, a significant prolongation of survival having been thus observed in the latter ($p<0.01$). With respect to the group to which the TGs from C3H-*gld* were co-transplanted along with the islets from C3H, the average number as above was 11 ± 3 and, accordingly, the survival of the transplanted islets was not prolonged. In addition, co-transplantation of the islets from C3H and the TGs from C3H to the C57BL/6-*lpr* did

not result in a significant prolongation of survival.

[Conclusion] The rejection of the transplanted pancreatic islets was suppressed by transplanting the islets together with testis grafts. It is inferred that the mechanism of such suppression is relied upon a reaction mediated by FasL on the grafts and Fas on the recipient.